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Conversion of a Linear to a Circular Plasmid in the Relapsing Fever Agent *Borrelia hermsii*

MEHDI S. FERDOWS,[1] PHILIP SERWER,[2] GARY A. GRIESS,[2]
STEVEN J. NORRIS,[3] AND ALAN G. BARBOUR[1]*

Departments of Microbiology and Medicine[1] and Department of Biochemistry,[2] University of Texas Health Science Center, San Antonio, Texas 78284, and Department of Pathology and Laboratory Medicine, University of Texas Medical School at Houston, Houston, Texas 77225[3]

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Spirochetes of the genus *Borrelia* have genomes composed of both linear and circular replicons. We characterized the genomic organization of *B. burgdorferi*, *B. hermsii*, *B. turicatae*, and *B. anserina* with pulsed-field gel electrophoresis. All four species contained a linear chromosome approximately 1 Mb in size and multiple linear plasmids in the 16- to 200-kb size range. Plasmids 180 and 170 kb in size, present in the relapsing fever agents *B. hermsii* and *B. turicatae* but not in the other two species, behaved as linear duplex DNA molecules under different electrophoretic conditions. A variant of strain HS1 of *B. hermsii* had a 180-kb circular instead of linear plasmid. There were no detectable differences in the growth rates or in the expression of cellular proteins between cells bearing linear forms and those bearing circular forms of the plasmid. The conversion to a circular conformation of monomeric length was demonstrated by the introduction of strand breaks with irradiation, restriction endonuclease analysis, and direct observation of the DNA molecules by fluorescent microscopy. Consideration of different models for the replication of linear DNA suggests that circular intermediates may be involved in the replication of linear replicons in *Borrelia* spp.

Spirochetes are a distinct phylum of eubacteria (41, 55). Known pathogenic members of this division are in the genera *Treponema*, *Borrelia*, and *Leptospira*. The genus *Borrelia* comprises microorganisms that are transmitted to vertebrates by hematophagous arthropods. Included in this genus are *B. hermsii* and *B. turicatae*, agents of relapsing fever in North America; *B. burgdorferi* sensu lato, the cause of Lyme disease; *B. anserina*, the agent of avian spirochetosis; and *B. coriaceae*, the putative agent of epidemic bovine abortion (5).

The genomes of *Borrelia* spp., like those of many other bacteria, are composed of a chromosome and plasmids. What distinguishes the *Borrelia* genome from that of most other bacteria is its largely linear structure. The chromosome of *B. burgdorferi* is a linear duplex DNA molecule about 1 Mb in length (6, 13, 14, 16, 17). Linear plasmids ranging in size from 5 to 55 kb are present in *B. burgdorferi* and *B. hermsii* (2, 32, 43, 54); These linear plasmids carry genes for outer membrane lipoproteins, including OspA, OspB, and OspD in *B. burgdorferi* (3, 4) and the serotype-specifying Vmp proteins of *B. hermsii* (31, 43).

Borrelia spp. also have circular DNA molecules, the form of extrachromosomal DNA in most bacteria (30, 51). Although these constitute only a small fraction of the *Borrelia* genome, circular plasmids carry what appear to be critical genes. In *B. burgdorferi*, these include *ospC*, which codes for an outer surface protein (35, 46), and homologs of *guaA* and *guaB*, which code for enzymes involved in de novo purine biosynthesis (36).

The structure of linear plasmids in *B. burgdorferi* has been the subject of detailed examination. Electron microscopic examination of the 49-kb linear plasmid of this species showed that the two strands of the duplex DNA are covalently closed at each end (4). The telomeres of the 16- and 49-kb plasmids of *B. burgdorferi* have been cloned, and sequence analysis of the telomeres revealed that there are short inverted repeats at their termini and that they have terminal hairpin loops (25, 28). A similar telomeric sequence was found on linear plasmids of *B. hermsii*, suggesting that other linear plasmids of this genus have similar structure (31). The telomeres of linear plasmids in *Borrelia* spp. have structural and sequence homology to those of some linear eukaryotic DNA viruses, specifically poxviruses and African swine fever virus (25).

B. hermsii and *B. burgdorferi* have several genome equivalents arrayed along the lengths of the wavy filamentous cells (32). Linear and circular plasmids have a copy number of approximately one to two per chromosome in both *B. burgdorferi* and *B. hermsii* (14, 26, 32). This arrangement suggests a tight coupling of plasmid and chromosome replication and segregation in borrelias.

To further understand the genetic organization of *Borrelia* spp., we examined the genomes of *B. hermsii*, *B. burgdorferi*, *B. turicatae*, and *B. anserina* in more detail. In the course of this characterization, we found linear double-stranded DNA molecules with sizes of 170 to 180 kb in *B. hermsii* and *B. turicatae*. In a strain of *B. hermsii*, the 180-kb linear plasmid spontaneously converted to a stably maintained circular conformation. We characterized both the circular and linear forms of this molecule. The existence of both circular and linear forms of the same DNA suggests possible modes of replication of the linear replicons of *Borrelia* spp.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Borrelia* species studied were *B. burgdorferi* B31 (ATCC 35210); *B. hermsii* HS1 (ATCC 35209) serotype 33 (formerly serotype C) (12); a *B. anserina* strain provided by R. Johnson, University of Minnesota, Minneapolis; and a *B. turicatae* strain originally provided by H. Stoenner, Rocky Mountain Laboratories, Hamilton, Mont. The borrelias were cultivated in polystyrene tubes containing 6 ml of BSK II medium as previously described (1). Cells were cloned by the method of limiting dilution as previously described (10). To estimate growth rate, borrelias at an initial con-

* Corresponding author. Phone: (210) 567-3938. Fax: (210) 567-6612. Electronic mail address: barbour@taliesin.uthscsa.edu.

centration of 2×10^6 cells per ml were grown in culture tubes. Growth at 34°C in a 1% CO_2 atmosphere was monitored visually and by cell counts every 12 h for 3 days. Spirochetes were counted in a Petroff-Hauser chamber by phase-contrast microscopy. *Escherichia coli* DH5 carried plasmid p7.16, a pBR322 derivative containing an insert of 15 kb of *B. hermsii* DNA and with a total size of 19.3 kb (43).

Preparation of DNA. Agarose blocks of genomic DNA were prepared as described previously (17). In brief, cells were harvested by centrifugation at 8,000 $\times$ *g* for 15 min at 20°C, washed with 150 mM NaCl–50 mM Tris (pH 8.0) (TN buffer) at 20°C, and then resuspended in TN buffer at a concentration of 10^9 to 10^{10} cells per ml. An equal volume of molten 1% low-melting-point agarose (Incert; FMC, Rockland, Maine) in TN buffer at 37°C was added to the cell suspension, and the mixture was poured into acrylic casting wells. Solidified agarose blocks were immersed in a lysis solution of 50 mM Tris (pH 8.0)–50 mM EDTA–1% sodium dodecyl sulfate (SDS), with 1 mg of proteinase K per ml (Boehringer Mannheim, Indianapolis, Ind.), and incubated at 50°C for 16 to 24 h.

DNA in *E. coli* was extracted by an alkaline lysis method (34). Supercoiled circular plasmids of *B. burgdorferi* were prepared as previously described (4). All plasmid preparations were further purified by ethidium bromide-CsCl density gradient centrifugation (34). Agarose blocks of *Saccharomyces cerevisiae* 334 chromosome were obtained from Beckman Instruments, Inc. (Palo Alto, Calif.). Agarose blocks that contained bacteriophage lambda linear multimers were obtained from FMC. The sizes of these DNA markers were those provided by the suppliers. In some experiments, the agarose blocks with DNA were first incubated in 10 mM Tris (pH 8.0)–1 mM EDTA (TE) buffer with 0.5 μg of ethidium bromide per ml for 1 h in the dark and then exposed for different lengths of time to 254-nm UV light (Ultraviolet Products, San Gabriel, Calif.) at an intensity of 330 $\mu W/cm^2$ at the block's surface.

TAFE. DNA blocks were washed three times for 30 min each with TE buffer and then loaded into wells of 1% agarose gels (Seakem GTG; FMC). The gels were then subjected to transverse alternating field electrophoresis (TAFE), a form of pulsed-field gel electrophoresis (17, 18). For TAFE, the buffer was 10 mM Tris base–0.5 mM free acid EDTA–4.5 mM glacial acetic acid (TAE). TAFE was performed in a Geneline system (Beckman) at 14°C with buffer recirculation. Unless otherwise noted, the electrophoresis parameters were a pulse time of 60 s, a constant current of 150 mA, a buffer volume of 3.5 liters, and a duration of 18 h. The gels were usually stained in ethidium bromide at a concentration of 0.2 μg/ml.

Two-dimensional agarose gel electrophoresis. A modification of the method of Serwer and Hayes was used for two-dimensional agarose gel electrophoresis (17, 50). DNA agarose blocks were loaded into the wells of a gel. For the first dimension, TAFE was performed as described above. For the second dimension, the gel was removed from the apparatus and placed in a horizontal gel electrophoresis unit such that the orientation of anode and cathode was perpendicular to the first-dimension electrodes. Constant field electrophoresis (6 V/cm) was then performed for 45 min in TAE buffer. The gel was stained with 0.2 μg of ethidium bromide per ml after the second electrophoresis.

Southern blot analysis. DNA bands were excised from TAFE agarose gels, extracted from the gel slices with a Gene Clean kit (BIO 101, La Jolla, Calif.), and then radiolabeled with [α-^{32}P]dATP with a commercial nick translation kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The procedure for Southern blot analysis was described previously (4). Briefly, the DNA in gels was subjected to acid (0.25 M HCl) depurination for 20 min prior to sequential NaOH denaturation, neutralization, and transfer to nylon membrane (34). Prehybridizations and hybridizations were performed at 37°C for 4 and 16 h, respectively. The hybridization solution consisted of 50% formamide, 6× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate), 5× Denhardt's solution, 0.5% SDS, 0.1 mg of denatured salmon sperm DNA per ml, and 0.1% sodium pyrophosphate. Final washes were performed at 64°C with 0.1× SSC–0.1% SDS–1 mM EDTA.

In situ restriction endonuclease digestion. Agarose blocks (100 μl each) were sequentially washed once in TE buffer, twice in TE buffer containing 1 mM phenylmethylsulfonyl fluoride, and then three times in TE buffer alone at room temperature for 30 min each. The blocks were equilibrated with 200 μl of restriction enzyme buffer for 30 min and then transferred to 100 μl of fresh buffer containing 10 to 30 U of restriction endonuclease. After the blocks were incubated in the enzyme for 6 to 24 h, they were heated at 68°C for 5 min. The molten sample was loaded onto a 0.7% agarose gel and then subjected to constant field gel electrophoresis in TBE buffer (90 mM Tris [pH 8.0], 90 mM borate, 2 mM EDTA) containing 0.2 μg of ethidium bromide per ml.

Fluorescence light microscopy of single DNA molecules. Agarose blocks were prepared from suspensions of 10^7 to 10^8 cells per ml of TN buffer as described above. The DNAs in the blocks were then subjected to in situ restriction endonuclease digestion followed by TAFE electrophoresis to remove linear DNA molecules. Agarose blocks were then removed from the gel wells and melted at 68°C. Ethidium bromide and β-mercaptoethanol were added to the molten agarose to final concentrations of 0.3 μg/ml and 5% (vol/vol), respectively. Microscopy of DNA molecules undergoing gel electrophoresis was then carried out (11). The apparatus for submerged horizontal agarose gel electrophoresis has been described by Griess et al. (22). One percent Seakem GTG agarose (FMC) in TAE buffer was used to cast an agarose gel (12 by 22 by 1 mm) in this apparatus. The DNA sample in molten agarose at 68°C was then gently loaded into the well of the agarose gels by use of a micropipette with the tip cut off. The gel was covered with a coverslip (24 by 50 by 0.2 mm), and the apparatus was placed on the stage of an Olympus BH2 epifluorescence microscope equipped with a BH2-DMG dichroic mirror filter cube (DM 570 with a BP 545 bandpass filter) (Olympus America, Inc., Lake Success, N.Y.). An electrical field of 6 V/cm was established across the electrodes of the gel apparatus. The direction of the electrical field was reversed by an external switch. For visualizing DNA, the ×100 oil immersion objective of the microscope was used. DNA molecules in the gel were illuminated with a green light and observed in red. The images were recorded with a Philips XX140/SP image intensifier (Philips Components, Slatersville, R.I.) coupled to a television monitor and a video cassette recorder (20). Still images were produced from videotapes by computer digitization with NIH Image, version 1.57 (45), and a Macintosh IIci microcomputer (Apple Computer, Inc., Cuptertino, Calif.) as described previously (21). Digitized images were used for photographic reproduction, contour length measurements, and counting of plasmids in different size groups. The factor for conversion of measured lengths from micrometers to kilobases was determined by measuring the lengths of circular plasmids of known sizes undergoing electrophoresis under similar conditions.

Two-dimensional protein electrophoresis. Late-log-phase cultures at 5×10^7 cells per ml of *B. hermsii* were centrifuged and washed with and resuspended in PBS (135 mM NaCl, 9 mM Na_2HPO_4, 6 mM KH_2PO_4 [pH 7.2]). For the first dimension, 2×10^8 cells were used in a nonequilibrium pH gradient electrophoresis (38, 39). The second dimension was SDS-polyacrylamide gel electrophoresis in 8 to 20% linear gradient polyacrylamide gels. Proteins in the gels were visualized by silver stain (23).

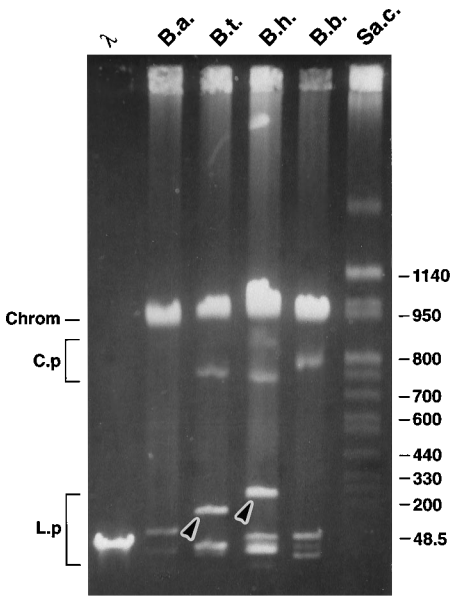

FIG. 1. TAFE of total DNA of bacteriophage lambda (λ), *B. anserina* (B.a.), *B. turicatae* (B.t.), *B. hermsii* (B.h.), *B. burgdorferi* (B.b.), and *S. cerevisiae* (Sa.c.). Locations of chromosomal bands (Chrom), circular plasmids (C.p) and linear plasmids (L.p) are indicated. Arrowheads mark the locations of the large plasmids in *B. turicatae* and *B. hermsii*. Sizes in kilobases of selected *S. cerevisiae* chromosomes (200 to 1,140 kb) and the linear form of bacteriophage lambda (48.5 kb) are indicated.

RESULTS

Genomic organization of different *Borrelia* species. Total genomic DNAs of *B. anserina*, *B. turicatae*, *B. hermsii*, and *B. burgdorferi* were subjected to TAFE (Fig. 1). Each species contained a DNA band that migrated with apparent size of about 950 kb relative to the *S. cerevisiae* chromosomal marker. In *B. burgdorferi* and *B. hermsii*, these bands have been identified as the linear chromosomes (6, 14, 16, 17, 32). When blocks containing less DNA were used, differences in the sizes of chromosomes of different *Borrelia* species were better discerned. The mean sizes (± standard error of the mean [SEM]) of chromosomes in four independent experiments were 985 ±

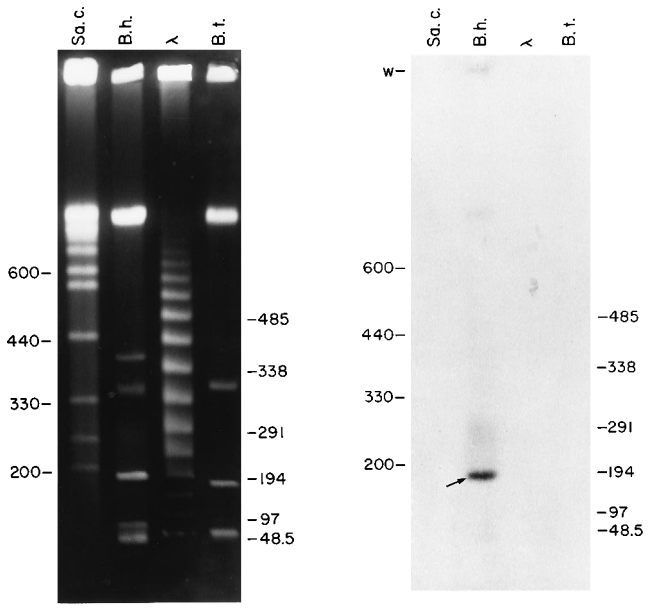

FIG. 2. Large plasmids of *B. turicatae* and *B. hermsii*. (Left) Ethidium bromide-stained TAFE agarose gel of *S. cerevisiae* chromosomes (Sa.c.), total DNA of *B. hermsii* (B.h.), bacteriophage lambda ladder (λ) and total DNA of *B. turicatae* (B.t.). (Right) Autoradiograph of Southern blot of the gel, which was probed with the ^{32}P-radiolabelled 180-kb DNA plasmid of *B. hermsii*. The numbers on the left and the right refer to selected sizes in kilobases of *S. cerevisiae* chromosome and lambda ladder concatemers, respectively. The TAFE parameters were a pulse time of 25 s, a current of 180 mA, and a duration of 18 h. The positions of the wells (w) and of the 180-kb linear DNA of *B. hermsii* (arrow) are shown.

12 kb for *B. anserina*, 1,012 ± 3 kb for *B. hermsii*, and 1,039 ± 9 kb for *B. turicatae*. The estimated chromosome size of *B. burgdorferi* was 960 ± 12 kb, which is consistent with the 950-kb size predicted by the physical map of chromosomes of this species (14, 16).

The four species were also distinguishable by other bands in the TAFE gels (Fig. 1). *B. turicatae*, *B. hermsii*, and *B. burgdorferi* had DNA bands that migrated with apparent sizes of 750 to 850 kb. The *B. burgdorferi* band with an apparent size of 800 kb in TAFE gels represents two supercoiled circular plasmids, 27 and 30 kb in size, which comigrate under these conditions (17, 26, 46). In *B. hermsii* and *B. burgdorferi* lanes, known linear plasmids in the size ranges of 16 to 55 kb were seen (2, 32, 43, 54). *B. anserina* and *B. turicatae* also had DNA bands in the size range of 15 to 90 kb; these presumably are the linear plasmids of these species. Of specific interest for the present study were DNA bands with relative migrations between 150 and 200 kb. These were uniquely present in lanes of total DNA from *B. turicatae* and *B. hermsii*. The next series of experiments defined the structure of these intermediate-sized bands in *B. hermsii* and *B. turicatae*.

Structural analysis of the DNA of *B. hermsii*. One feature distinguishing linear from supercoiled circular duplex DNA is the change in electrophoretic mobility of the linear molecules with changes in pulse duration in pulsed-field gels (9, 17). Whereas the mobility of linear molecules is pulse time dependent, the migration of supercoiled circular molecules is independent of the pulse duration (9). Consequently, variations in pulse time result in changes in the apparent sizes of supercoiled circular DNA relative to those of linear DNA. Figure 2 (left panel) shows the TAFE patterns of total *B. hermsii* and *B. turicatae* DNAs when electrophoresis was performed at a pulse time of 25 s instead of the 60-s pulse time of Fig. 1. Linear lambda DNA concatemers and *S. cerevisiae* chromosomes were the size standards. Under these conditions, *B. hermsii* and *B. turicatae* DNA bands, which migrated in the size range of 750 to 850 kb under the conditions shown in Fig. 1, had apparent sizes in the range of 340 to 430 kb compared with those of the *S. cerevisiae* chromosomes and lambda multimers. This apparent change in sizes of bands with respect to the linear size standards and in response to pulse time variation would be expected if the DNA molecules were supercoiled circular plasmids (9, 17, 19). In contrast, *Borrelia* DNA bands in the range of 150 to 200 kb in Fig. 1 migrated in the same size relationships with the linear size standards when the pulse time changed, a finding consistent with a linear duplex structure of these *Borrelia* molecules (9). With lambda multimers as size markers, we estimated the sizes (± SEM) of the large plasmids to be 170 ± 1 and 180 ± 1 kb for *B. turicatae* and *B. hermsii*, respectively.

Plasmid linearity was confirmed by a second method. Electrophoresis of *B. burgdorferi* DNA in TAFE gels in the presence or absence of the intercalating agent ethidium bromide distinguishes between linear and supercoiled circular DNA (17). Agarose blocks containing *B. hermsii* DNA were subjected to TAFE in the presence or absence of ethidium bromide (Fig. 3). Supercoiled circular plasmid controls were the 27- and 30-kb plasmids of *B. burgdorferi* B31, which comigrate in TAFE gels under these conditions, and a 19.3-kb plasmid from *E. coli*. The migrations of these supercoiled circular plasmids relative to that of the linear chromosomes of *S. cerevisiae* were affected by the presence of ethidium bromide in the buffer. Whereas these supercoiled plasmid controls had apparent sizes in the ranges of 740 and 650 kb, respectively, in the absence of ethidium bromide (Fig. 3, left panel), their apparent sizes in the presence of ethidium bromide were, correspondingly, 570 and 510 kb (right panel). In contrast, and as predicted for linear DNA, the migration of the 180-kb DNA band of *B. hermsii* relative to that of yeast chromosomes was unaffected by the presence of ethidium bromide. Similar findings were obtained with the 170-kb plasmid of *B. turicatae* (data not shown).

These experiments indicated that the 170- to 180-kb DNAs

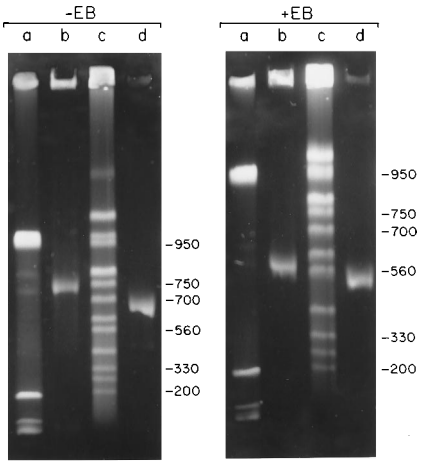

FIG. 3. TAFE migration of total DNA of *B. hermsii* in the absence (−) and presence (+) of ethidium bromide (EB). Shown are *B. hermsii* (lane a), supercoiled plasmids (27 and 30 kb) of *B. burgdorferi* (lane b), *S. cerevisiae* chromosomes (lane c), and supercoiled plasmid p7.16 (19.3 kb, lane d). Numbers to the side refer to sizes in kilobases of selected *S. cerevisiae* chromosomes.

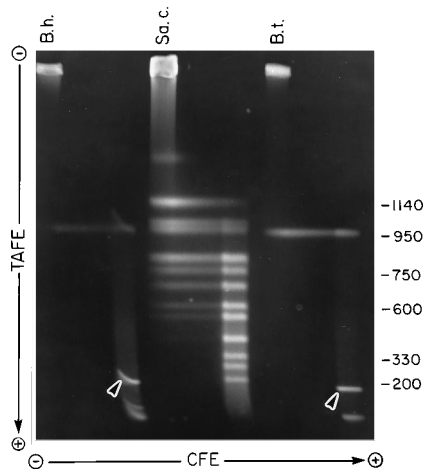

FIG. 4. Two-dimensional gel electrophoresis of total DNA of *B. hermsii* (B.h.), *S. cerevisiae* (Sa.c.), and *B. turicatae* (B.t.). The first dimension was TAFE, and the second dimension was constant field electrophoresis (CFE). Sizes in kilobases of selected *S. cerevisiae* chromosomes are indicated on the right. Arrowheads indicate the large linear plasmids in *B. hermsii* and *B. turicatae*.

of *B. turicatae* and *B. hermsii* were not supercoiled circular plasmids. With two-dimensional agarose gel electrophoresis (Fig. 4), we addressed the possibility that these intermediate-sized DNA molecules were open circles. For the first dimension, agarose blocks of total DNA were loaded on the gel and subjected to TAFE. A high constant field was then applied in a direction perpendicular to that of the first. An open circular DNA molecule may enter the gel in the first dimension, but its migration will be arrested in the second dimension (17, 49). In this experiment, the 180-kb DNA of *B. hermsii* and the 170-kb band of *B. turicatae* behaved like the linear chromosomes of *S. cerevisiae* in their migrations in both dimensions (Fig. 4). As was noted previously (48), linear DNA with a size of about 500 kb or greater was partially or totally arrested in its migration in the second dimension.

We next determined whether the 180-kb linear DNA of *B. hermsii* represented either a multimer of a smaller linear plasmid or a large separated fragment of the chromosome. The 180-kb DNA band was isolated from the gel, labeled, and used as a probe in a Southern blot of the gel shown in the left panel of Fig. 2. The right panel of the figure shows the resultant autoradiograph. The labeled 180-kb DNA band of *B. hermsii* hybridized to itself but not detectably to other DNA molecules of *B. hermsii* or to the 170-kb linear plasmid of *B. turicatae*. Further evidence that the 180-kb band was not a multimer was obtained with restriction endonuclease digestion of the 180-kb DNA. If the large replicon was a multimer of a smaller plasmid, one would expect that the sizes of the restriction fragments would add up to less than 180 kb. Digestion with *Cla*I of the 180-kb plasmid yielded fragments with sizes of 37, 34, 33, 18, 15, 14, 12, 9, 6, and 4 kb (data not shown). A cumulative size of 182 kb indicated that the plasmid was a monomer.

A 180-kb circular plasmid in *B. hermsii*. In the course of these studies, we observed an isolate of *B. hermsii* HS1 with a variation of the genomic organization described above. The variant was cloned by limiting dilutions and was designated HS1C to distinguish it from the isolate of HS1 described in the experiments above; for subsequent experiments, the latter isolate was designated HS1L. HS1C differed from HS1L in apparently lacking the 180-kb linear plasmid (Fig. 5). HS1C grew at a rate indistinguishable from HS1L: the mean generation times (± SEM) were 7.1 ± 0.2 h for the parent and 7.3 ± 0.2 h for the variant. Furthermore, a two-dimensional analysis of total cellular proteins failed to demonstrate any differences between HS1C and HS1L in their detectable proteins of *B. hermsii* (data not shown). If the DNA of the 180-kb plasmid, which is approximately 15% of the total DNA of the cell, had been lost from the cell, one would expect to find differences in the protein patterns. The finding of no detectable differences suggested either that the DNA of the 180-kb plasmid was in a different plasmid conformation or that it was integrated into the chromosome in HS1C.

To distinguish between these possibilities, we probed the Southern blot of the TAFE gel of Fig. 5 with the 180-kb plasmid DNA. The probe hybridized as expected to the linear molecule with a size of 180 kb in HS1L but predominantly to the well area in the lane of DNA of the variant HS1C (Fig. 5, right panel). Under these electrophoretic conditions, the chromosomes of both isolates migrated into the gel. There was no hybridization of the probe to the chromosome of HS1C, and, thus, there was no indication that all or part of the 180-kb linear plasmid was part of the chromosome.

To further define the conformation of hybridizing DNA retained in the well, we used ethidium bromide and UV irradiation to introduce random double-stranded breaks. This would convert a circular DNA molecule to a linear conformation, thereby allowing its migration into the gel's matrix. Blocks containing total genomic DNA of either HS1L or HS1C were incubated with ethidium bromide, irradiated for different lengths of time, and then subjected to TAFE (Fig. 6). Breakage of DNA in the agarose blocks was a function of the time of UV light irradiation. This amount of breakage caused the disappearance of hybridizable material from the well of HS1C DNA and the appearance of a hybridizing band with a size of 180 kb, a finding that is consistent with a circular nature of the 180-kb plasmid in HS1C.

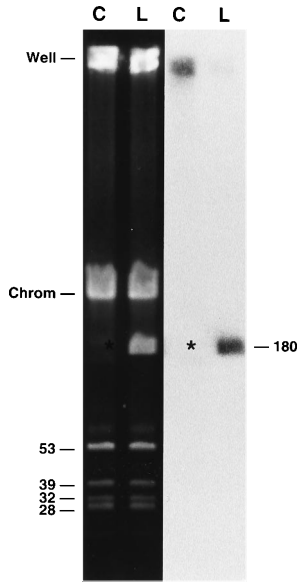

FIG. 5. TAFE gel (left panel) and Southern blot (right panel) of total DNA of *B. hermsii* HS1L (L) and the variant HS1C (C). Numbers refer to the sizes of linear plasmids in *B. hermsii* HS1L. The asterisk marks the location of the 180-kb linear plasmid. The Southern blot was probed with nick-translated 180-kb linear plasmid. The positions of the wells and of the chromosome (Chrom) are indicated. The TAFE parameters were a current of 180 mA and consecutive pulse times of 1 s for 9 h and 9 s for 9 h.

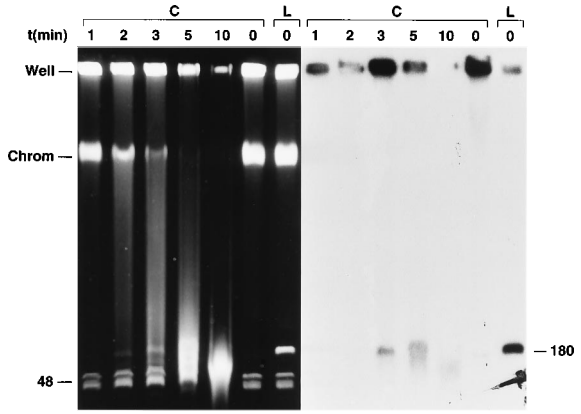

FIG. 6. UV-induced breakage of DNA. (Left) Ethidium bromide-treated agarose blocks of *B. hermsii* HS1L (L) and the variant HS1C (C) were exposed to UV light for various lengths of time (t) in minutes, as indicated, and then subjected to TAFE. (Right) Autoradiogram of Southern blot of the gel probed with the radiolabeled 180-kb linear plasmid. The positions of the wells (W) and of the linear chromosome (Chrom) are shown. Sizes in kilobases of the large linear plasmid and bacteriophage lambda DNA are indicated.

To further test this hypothesis, we digested DNA in the blocks with restriction endonucleases and probed the resultant fragments with a radiolabeled 180-kb linear plasmid. When linear and circular conformations of a plasmid DNA are cut by the same restriction endonuclease, the total number of fragments generated by digestion of the linear form is one more than that obtained by the digestion of the circular form. When DNA of HS1C was digested with *Eco*RI or *Bgl*II, an additional restriction endonuclease fragment was seen (Fig. 7). In the *Eco*RI digest of HS1C DNA, the probable junctional fragment was identified. This fragment with a size of about 34 kb in the circular form may result from the fusion of the two putative terminal fragments with sizes of 28 and 6.0 kb of the linear form.

Fluorescence microscopy of plasmid molecules in an electric field. The preceding experiments could not distinguish between monomeric, multimeric, and concatenated circular forms of this DNA. To provide this discrimination, we used

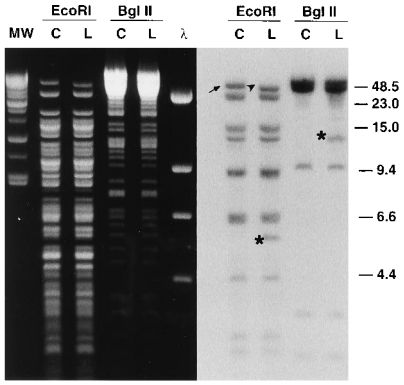

FIG. 7. Restriction fragment length polymorphisms of the 180-kb DNA of *B. hermsii* HS1L (L) and the variant HS1C (C). (Left) Agarose gel electrophoresis of *Eco*RI and *Bgl*II fragments. (Right) Autoradiogram of the Southern blot of the gel. The blot was probed with the radiolabeled 180-kb plasmid of *B. hermsii*. The asterisks mark additional bands present in restriction fragments of HS1L DNA. In *Eco*RI digests, the arrow points to the putative junctional fragment formed from the two ends of the linear plasmid (arrowhead and asterisk). Molecular weight standards from Gibco/BRL and lambda *Hin*dIII fragments were the size standards.

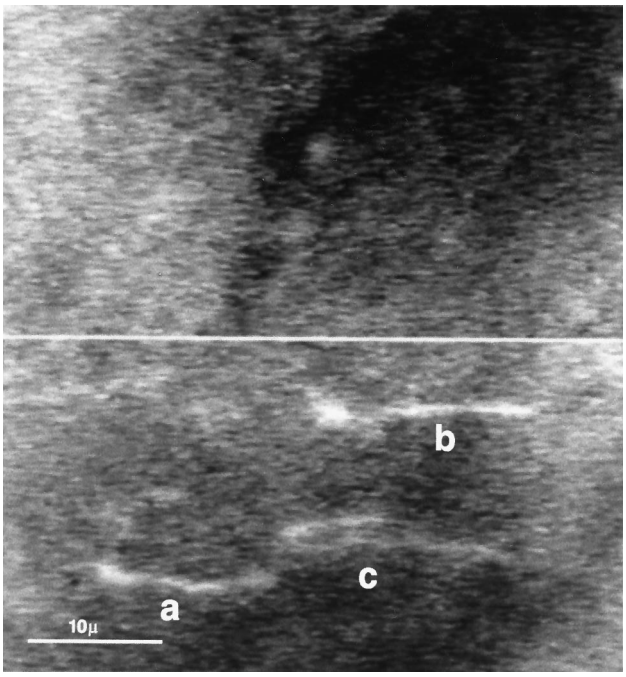

FIG. 8. Digitized fluorescence microscope images of circular DNA molecules of *B. hermsii* HS1C undergoing gel electrophoresis from left to right. The bottom frame shows circular plasmids a, b, and c anchored on agarose spikes inside the gel. DNA molecules are elongated in the direction of the applied electrical field. Molecule c is curved around the spike. The top frame shows, as a control, background fluorescence without plasmid DNA.

fluorescence microscopy with an image intensifier to directly visualize individual DNA molecules. Individual DNA molecules, stained with ethidium bromide and undergoing agarose gel electrophoresis, have been observed with a fluorescence microscope (52). During migration through the gel matrix, linear DNA molecules often transiently wrap around obstacles and form a U-shaped conformation. As electrophoresis continues, they slide off the obstacles and assume a more relaxed random coil conformation. In contrast, open or slightly coiled circular DNAs become permanently caught on the obstacles as long as a constant and strong field is applied (33, 53). This conformation-dependent behavior of DNA molecules was exploited to study the circular DNA molecules of *B. hermsii.*

To minimize the presence of linear chromosomal and plasmid DNA and to enrich for circular DNA in the microscopic fields, agarose blocks of genomic DNA were digested first with *Eag*I. (Preliminary study of infrequently cutting restriction endonucleases indicated that *Eag*I cuts the chromosomal DNA but not the plasmids of *B. hermsii* HS1.) The blocks were then subjected to TAFE to remove linear DNA molecules. This left the circular plasmids entrapped in the agarose block, as demonstrated in Fig. 5. The DNA molecules were then observed by fluorescence light microscopy. Figure 8 is an example of some of the DNA molecules that remained entangled on the agarose. The molecules exhibited behavior characteristic of open or slightly coiled circles (33, 53). That is, when the field was on, DNA molecules became threaded on spurs of agarose and were anchored to the gel at one end. The rest of the DNA molecule stretched in the direction of the applied field. When the electric field was removed, the molecules condensed around the attachment site. Reversal of the direction of the electrical field caused the majority of these molecules to realign themselves with the direction of the new field while still

anchored to the gel. However, when the electrical field was reversed, some molecules were freed, presumably by flipping off the agarose obstructions, and traveled in the direction of the new field for some distance before getting caught on a new obstruction.

Quantitative examination by fluorescence microscopy revealed a range of circular DNA sizes of between 10 and 200 kb in HS1L and HS1C. For further comparison of the two isolates, circular DNA molecules in two different size groups from three microscopy experiments were counted. In one group, circular DNA sizes were between 10 and 60 kb (134 plasmids for HS1L and 129 plasmids for HS1C). In the second group, the DNA sizes were in the range of 100 to 200 kb (51 plasmids for HS1L and 111 plasmids for HS1C [$\chi^2 = 14.7$, $P < 0.001$]). The 180-kb plasmid would be in this size range. Significantly more circular DNA molecules in the size range of 100 to 200 kb were observed in the DNA samples of *B. hermsii* HS1C; there was no difference between isolates in DNA of smaller sizes. Larger DNA circles, which might include either multimers of the 180-kb plasmid or concatenated circular forms of this plasmid, were not observed in either HS1C or HS1L. The identities and origins of all of the circular DNA molecules observed by fluorescence microscopy are not known at this time. HS1L had molecules behaving like circles in the gels, but not as many as HS1C had in the larger size range.

DISCUSSION

In this study, we first characterized the genomic DNA profiles of four *Borrelia* species by pulsed-field gel electrophoresis. Our results indicate that the chromosomes of these species are linear and are about 1 Mb in length. The linearity and size of the chromosome of *B. burgdorferi* have been well described (6, 13, 14, 16, 17, 40). There was also previous evidence that chromosomes of *B. hermsii*, *B. turicatae*, and *B. anserina* behave as linear molecules in pulsed-field gels (14, 32, 35). This report confirms those findings. What we uniquely describe are linear plasmids with sizes of greater than 100 kb in some *Borrelia* species. DNA molecules with sizes between 150 and 200 kb had been reported in *B. duttoni*, *B. coriaceae*, and *B. burgdorferi* (14, 24, 42). However, the structures of these large plasmids or the chromosomes were not further investigated. The 180-kb plasmid of *B. hermsii* can exist and replicate as either a linear or circular molecule. No genes have yet been assigned to the 180-kb plasmid; presumably there are several. In any case, conversion of this plasmid to a circular conformation did not detectably alter the expression of genes in the cell. If any DNA is missing from the circular 180-kb plasmid in comparison to its linear embodiment, it was not detectable in this study.

The finding of both circular and linear versions of the same DNA offers insights into how plasmids replicate in *Borrelia* species. For linear replicons, the telomeres would seem to be essential for both replication and stability: replication must maintain the structure of telomeres. The telomeres of the *B. burgdorferi* linear plasmids characterized to date consist of a short inverted terminal repeat in which the two strands of the DNA are connected by four unpaired nucleotides that form a hairpin loop (25, 28). Nearly identical sequences exist at the terminus of a *B. hermsii* linear plasmid (31). The telomeres of *Borrelia* linear plasmids have structural similarities to the telomeres of African swine fever virus and poxviruses (25). Therefore, models for the replication of poxviruses provide a starting point for considering the replication of the linear plasmids of *Borrelia* spp.

Poxvirus telomeres are AT-rich loops terminating long inverted repeats. The terminal hairpin loops exist in two equimolar forms of inverted sequence, referred to as "flip-flop" structures (7). A nick near the hairpin loop occurs during viral growth in the cell (44). Replicating viruses have intermediates with both head-to-head and tail-to-tail junctions of termini (37). These findings led to different models of poxvirus replication (7). In one model, which is similar to the model for replication of eukaryotic telomeres (8, 15), the termini are nicked by a site-specific nuclease and the hairpins are opened. The resulting 3′ overhangs at the ends are used to synthesize the new telomeric palindrome. Replication then proceeds through self-priming at each end of the DNA molecule without the formation of any junctional fragments. In a variation of this model, the process of nicking, synthesis of new palindrome, and self-priming occurs at one end only. When the replication goes through the hairpin of the opposite end, head-to-head or tail-to-tail junctions are produced. Resolution of intermediates can result in flip-flop of the hairpin sequences. In either case, circular intermediates would not be predicted and head-to-tail junctions are not expected to form unless there is concatemerization of opened ends of monomers.

Another model of poxvirus replication calls for a circular DNA intermediate. According to this model, replication would begin from a single origin somewhere along the length of the linear replicon. This would lead to a palindromic circular dimer with head-to-head and tail-to-tail junctions. Resolution into two monomeric linear DNAs occurs by a site-specific nuclease that nicks palindromic sequences near the junction. Structural variations, i.e., flip-flop structures, at both ends of the linear DNA can be obtained by resolution of the circular dimer through rearrangement and separation of the daughter strands. This model predicts that a replicative intermediate of the 180-kb linear plasmid of *B. hermsii* would be a circle 360 kb in size. Circles of this size were not seen in either HS1L or HS1C by fluorescence microscopy.

The third model of replication of poxvirus replication is most consistent with the findings of the present study (Fig. 9). According to this model, an intermediate in the replication process is monomeric circular DNA, which results when the linear DNA with complementary 5′ and 3′ overhangs, after opening of the terminal hairpin, circularizes to form a head-to-tail junction. The circular intermediate could then replicate by mechanisms common to other circular replicons. The circular intermediates would then be resolved into linear DNA molecules by a site-specific nuclease that recognizes a sequence in the terminal inverted repeats. This model, in contrast to the first two models, requires that the left- and right-terminal hairpin loops always be complementary.

In *B. burgdorferi*, linear plasmids have short inverted terminal repeats (26, 28). Inverted and complementary sequences (flip-flop) have not been detected at either end of a linear plasmid (28). Junctional fragments composed of the right and left ends of the linear plasmid, i.e., head to tail, have been detected (27, 29). Linear plasmids of *B. hermsii* appear to have a similar structure (31). If the circular plasmid of HS1C is actually a stable form of the putative replicative intermediate, then the replicative intermediate is a monomer and not a dimer. The circular form of the 180-kb plasmid may have arisen as a result of the failure of the site-specific nuclease to recognize the telomeric palindromes, possibly due to either a point mutation in the recognition sequence for the putative endonuclease or an alteration in the site-specific nuclease or other *trans*-acting function. The answer to these questions awaits sequence analysis of the termini of the HS1L 180-kb linear plasmid and the junctional fragment of the circular DNA in HS1C.

Finally, we propose that a more appropriate designation for

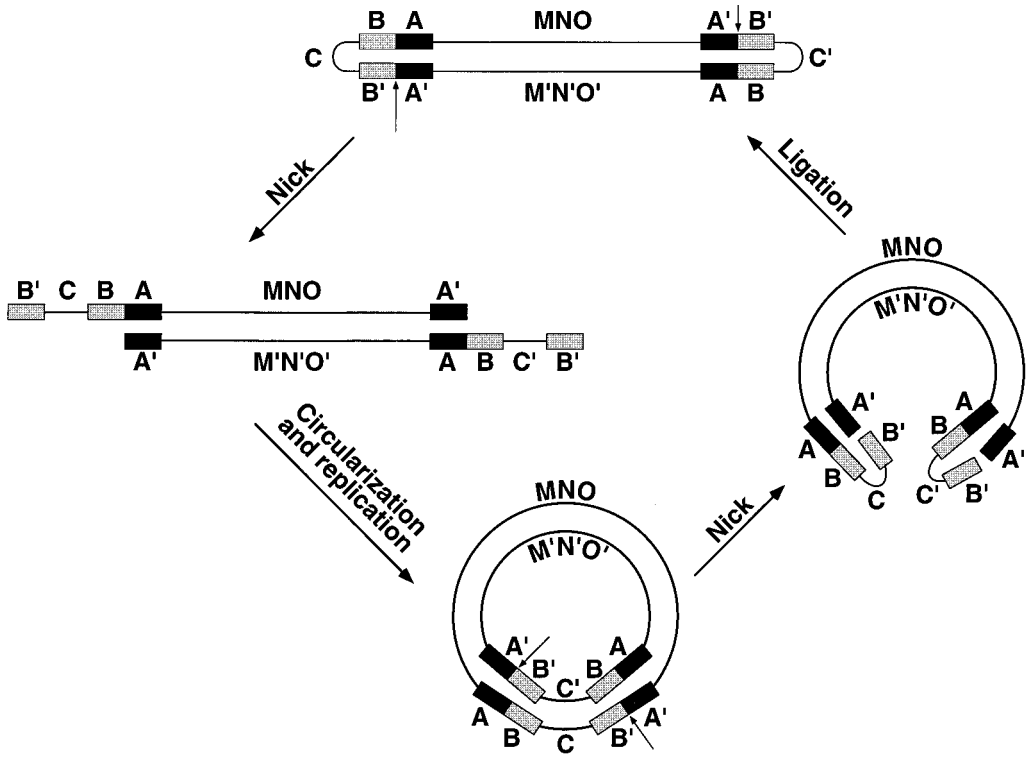

FIG. 9. Model for replication of a linear plasmid of a *Borrelia* sp. through a monomeric circular intermediate. At the top of the figure is a hypothetical duplex linear plasmid with telomeres (C and C′), inverted terminal repeats (BA and A′B′), and selected sequences in the middle of the replicon (MNO and M′N′O′). A nick introduced in the inverted repeat opens the ends of the plasmid (left). The ends are complementary, and thus the plasmid could circularize and replicate in this form (bottom). The same or a second nicking process could produce a double-stranded break in the circular molecule. Inasmuch as the free single-stranded ends of this latter structure are complementary, linear molecules with hairpins could be reconstituted (right).

most if not all of the plasmids in *Borrelia* spp. is minichromosomes (3, 17). This suggestion is prompted by these observations: (i) the presence of both a linear chromosome and linear plasmids in *B. burgdorferi* (3, 47); (ii) the siting of genes for major outer membrane proteins and of important biosynthetic genes like *guaA* and *guaB* on plasmids (36); (iii) the identical copy number for chromosomes and plasmids in the cell (14, 26, 32); and, as demonstrated here, (iv) the presence in the cells of linear and circular replicons as large as 180 kb, about one-fifth the size of the chromosome. It is conceivable that previously identified circular plasmids in *Borrelia* spp. derive their present structure from linear plasmids as the result of an event similar to that described here. Alternatively, circular plasmids may predate linear plasmids in *Borrelia* spp. or their predecessors; linear replicons have not been found in other spirochetal genera examined to date. In either case, circular and linear plasmids of *Borrelia* spp. may share a common replication mechanism.

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